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Surface Charge and Overlayer pH Influence the Dynamics of Supported Phospholipid Films

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Abstract

Understanding the thermodynamics and kinetics of interactions between model lipid bilayers and planar supports is of critical importance in the furtherance of biosensing and the creation of biomimetic devices. Evaluating these properties can be accomplished through understanding the diffusional properties of the bilayer constituents. In this report, the dynamics of a model DMPC bilayer supported on a phosphorylated silica surface are studied in the presence and absence of interfacial Ca^{2+} as a function of pH of the aqueous overlayer. The data for this system reveal the importance of the balance of ionic interactions between the interfacial species, and the dependence of the diffusional, kinetic and thermodynamic properties of the system on pH. The thermodynamic data suggest that interactions between the bilayer and surface are mediated enthalpically rather than entropically.

Graphical Abstract

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Keywords

Translational Diffusion; Supported Bilayer; Interfacial Complexation; Calcium Ion Binding

Introduction

Lipid bilayers have been investigated extensively because of their central importance in cellular function and for the role they play in mediating the transport of therapeutic agents into cells. The plasma membrane is a highly complex structure, despite its molecular thickness, owing to the presence of *ca.* 1500 unique molecular constituents.[1] These various species are not only distributed non-uniformly within the plasma membrane, but in many instances the inner and outer leaflets are asymmetric in living cells.[2, 3] There is a great deal of structural and dynamic information that remains to be elucidated before the function of the plasma membrane is understood fully.

Despite the complexity of plasma membranes, systems have been constructed with the goal of mimicking their function.[4, 5] Such biomimetic bilayer structures are studied most frequently either in the form of unilamellar vesicles or as supported planar bilayers. The structural format of the latter does not incorporate the curvature that is characteristic of a living cell, thereby limiting the correspondence between such systems and the plasma membrane. That issue notwithstanding, planar supported bilayers are studied frequently because of the utility of this structural format for experimental investigations and the construction of sensing systems.

Recent work has focused on the influence of both pH and divalent cations during the formation of the supported lipid membrane framework. The use of Ca^{2+} has been found to be important for both enhanced stability of the ruptured vesicles during self-assembly and post-formation via added adhesion strength.[6–8] The addition of metal cations during self-assembly is thought to increase rupture and adhesion strength of the lipid vesicles through increased charge screening between lipid and support. Similar to the addition of metal cations, pH has been found to be an effective means of controlling the formation of self-assembled supported lipid membranes by means of modifying the electrostatic charge screening between the lipid vesicles and supporting surface.[9, 10] Despite these advancements, there is limited knowledge of how pH and surface charge influence lipid dynamics post-assembly.

In this work we have focused on planar supported bilayer structures because, ultimately, we are interested in developing biomimetic sensing systems capable of housing transmembrane proteins. With this goal in mind and recognizing the fact that bilayer fluidity is one essential physical property for the proper function of transmembrane proteins, we have undertaken studies to evaluate the molecular structural issues that are of primary importance in mediating supported bilayer dynamics and adhesion. To quantitate the dynamics of such systems, we use fluorescence recovery after photobleaching (FRAP) measurements of fluorescent probe molecules incorporated into the model bilayer structures. We report here on the study of model phospholipid bilayers supported on a (modified) silica support, where three experimental variables are controlled systematically. The first is the location and

identity of the probe molecule; the second is the chemistry controlling the interaction between the phospholipid and the (phosphorylated) silica support and the third is the pH of the overlayer. The evaluation of the role of these variables provides significant insight into the chemical factors that are of greatest importance in controlling model bilayer dynamics.

Experimental

Materials

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1-(12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl)-2-hydroxy-*sn*-glycero-3-phosphocholine (NBD-LPC) were purchased from Avanti Polar Lipids, Inc. Chloroform was purchased from Macron Fine Chemicals and used as received. 2,4,6-collidine (99%), acetonitrile (HPLC), calcium chloride dihydrate (99%), and phosphorus oxychloride (99%), were purchased from Sigma Aldrich and used as received. Sodium chloride was used to maintain constant ionic strength in a 10 mM citric acid-sodium phosphate buffer (ACS grade). The buffer was used to maintain pH and tested prior to use in all experiments using a pH meter (Thermo Fisher). Water was obtained from a Barnstead water purification system and used for all experiments.

Substrate Phosphorylation

The round cover-glass substrates were composed of D263M borosilicate glass (#1.5 x 25 mm dia., Warner Instruments) and were placed in piranha solution ($1\text{H}_2\text{O}_2:3\text{H}_2\text{SO}_4$, *Caution! Strong oxidizer!*) for 30 min. After cleaning, the substrates were sonicated for 5 minutes with water (three times with fresh water for a total of 15 min.) and thoroughly rinsed with dry acetonitrile. The substrates were then exposed to phosphorus oxychloride (0.4 mL) and 2,4,6-collidine (0.4 mL) in 10 mL dry acetonitrile for 3 hrs. The substrates were rinsed thoroughly with dry acetonitrile and kept covered in water until needed for spin-coating.

Formation of DMPC/NBD-LPC Supported Bilayers

Phospholipid bilayers were prepared using a spin coating method described previously.[11, 12] Briefly, the circular cover-glass was rinsed thoroughly with water and dried under N_2 . 150 μL of a 1 mg/mL DMPC/NBD-LPC (2 mol%) or DMPC/perylene (2 mol%) in chloroform solution was added to the cover-glass. Immediately after the surface was wetted, the spin-coater (EC101D, Headway Research, Inc.) was accelerated to 3000 rpm for 60 sec. The substrate was then covered in a petri dish and kept in the dark until measured no more than 24 hrs after spin-coating.

Optical Ellipsometry

Optical ellipsometry measurements were carried out on a J. A. Woollam Co., Inc. model M-44 spectroscopic ellipsometer with a wavelength range of 414 nm to 736.1 nm, utilizing 44 wavelengths for simultaneous measurements at an angle of incidence of 75° . For all ellipsometric measurements, the DMPC films were first prepared on Si(100) wafers (University Wafer). Prior to film deposition, optical constants on freshly cleaned Si and phosphorylated-Si surfaces were determined at the 44 wavelengths used by the ellipsometer. In both cases, the refractive index of the DMPC film was set to 1.50.[13] A home-built cell

with quartz windows was used for all measurements performed under an aqueous adlayer. Optical constants of water as a function of wavelength were calculated using a Cauchy layer in coordination with constants from the literature.[14, 15]

Fluorescence Recovery After Photobleaching (FRAP)

FRAP measurements were conducted on a Nikon C2+ confocal laser scanning microscopy system described in detail elsewhere.[16] Briefly, the NBD-LPC was excited with the 488 nm line of a diode laser, and emission was detected at 525 nm using a standard PMT detector. A 20x objective lens was used for the films. Two independently prepared DMPC films were measured with a minimum of 5 measurements at random locations for each pH. Initial intensities (I_{pre}) were measured for 10 sec. The bleaching time was set to 2 sec. at 100% laser power, and the recovery signal after photobleaching was collected over 2 min. Temperature studies were performed using a home-built microscope stage heater.

To account for acquisition bleaching, the recovery signals, $I_{frap}(t)$, were normalized using double normalization described by Phair *et al.* (Eq. 1).[17] $I_{frap-pre}$ is the average stimulation region intensity prior to photobleaching, $I_{ref-pre}$ is the average reference intensity prior to photobleaching, $I_{ref}(t)$ is reference intensity after photobleaching, and $frap(t)$ is the calculated normalized intensity.

$$frap(t) = \frac{I_{ref-pre}}{I_{ref}(t)} \cdot \frac{I_{frap}(t)}{I_{frap-pre}} \quad (1)$$

XPS measurements

XPS measurements were performed on a Perkin-Elmer Phi 5600 ESCA instrument described previously.[16] Survey scans of 0 1100 eV binding energy and detailed scans for Ca2p, C1s, O1s, P2p, and Si2p were measured for each sample. All peaks were corrected in reference to the C1s peak associated with adventitious C at 284.8 eV. Peak areas were determined by Shirley background subtraction and an area correction using calibrated sensitivity factors.

Results and Discussion

A primary purpose of this work was to evaluate the effect of surface charge and functionality on the fluidity and heterogeneity of a supported DMPC bilayer. The DMPC bilayers are supported on a phosphorylated silica surface in the absence or presence of adsorbed Ca^{2+} ions, resulting in a supporting interface with differing surface charge density. Examining changes in the dynamical properties of the supported bilayers using fluorescence recovery after photobleaching (FRAP), allows for the direct examination of the effect of surface charge on changes in fluidity and heterogeneity of the system. Further examination, via changes in overlayer pH, allows for a broader understanding of how the dynamical behavior of these films is mediated by charge. Two fluorescent probes (perylene or NBD-LPC) were mixed with DMPC during bilayer preparation and used for the fluorescence measurements. A comparison between a fluorescent probe interacting with the interface due to tethering to

the lipid acyl chain (NBD-LPC) and a non-interacting fluorescent probe residing within the acyl chain region (perylene) of the bilayer is presented.

Preparation of supported DMPC bilayers

The standard borosilicate glass supports used in this work were reacted with phosphorous oxychloride and 2,4,6-collidine to produce a phosphorylated surface. The DMPC films were deposited on the phosphorylated surface using the spin-coating method.[11, 12] Optical ellipsometry was used to measure the thickness of the resulting lipid adlayer both in air after spin-coating, and under a buffered (pH 5.0) aqueous overlayer (Table 1). The measured thickness of the DMPC films formed on a phosphorylated surface and containing either perylene (P/PO₃) or NBD-LPC (NBD/PO₃) in air, and under an aqueous adlayer, are consistent with the formation of a DMPC bilayer.[13, 18]

Interaction of Ca²⁺ with the phosphorylated surface

XPS was utilized to provide a semi-quantitative assessment of the Ca²⁺ adsorbed on the surface following bilayer preparation. An idealized schematic of the supported film surface for the systems studied here is shown in Fig. 1. During the reaction of the phosphorylated surface with Ca²⁺, an increase in deposited calcium is seen by XPS (Fig. 2). Characterizing the amount of Ca²⁺ present is accomplished ratiometrically. The ratio of the Ca2p peak to the SiO₂ Si2p peak is *ca.* 0.05 following deposition. For both the phosphorylated and calcium-containing phosphorylated surfaces, no phosphorus P2p peak was seen. The Ca:Si ratio is slightly higher than that seen for the analogous interface prepared by Langmuir-Blodgett deposition, and slightly less than phosphorylated surfaces prepared on gold.[16, 19] This is not a surprising result owing to the different type and extent of ordering for the gold and silica surfaces. While XPS provides only a semi-quantitative assessment of the relative abundance of metal ions at a surface, the surface density of calcium is sufficient to influence the dynamics of the supported bilayer (*vide infra*).

Bilayer diffusion dynamics mediated by Ca²⁺ and adlayer pH

Fluorescence recovery after photobleaching (FRAP) experiments were performed on the supported zwitterionic phospholipid bilayers in the presence and absence of adsorbed calcium ions (Ca²⁺). In addition, the pH of the aqueous overlayer was examined as a variable to gauge the role of pH in mediating the order and fluidity of the bilayer. Data for typical recovery curves and images of the time-lapse series during the FRAP experiment for a bilayer containing the fluorescent probe NBD-LPC supported on the calcium phosphorylated surface are shown in Fig. 3 and on the phosphorylated surface without calcium are shown in Fig. 4. The recoveries exhibit different functional forms that depend on the composition of the interface (Fig. 1).

The detailed information content of the FRAP recovery curves requires a framework for interpretation. For the interfaces considered here, with the tethered chromophore NBD, the functional form of the recovery is related to the reaction diffusion model described by Sprague *et. al.* in Eq. 1.[20, 21]

In this model F represents the concentration of freely diffusing NBD-LPC molecules, M is the concentration of available binding sites on the calcium-phosphorylated or phosphorylated surface, and B is the complex formed between the NBD-LPC and the surface binding site. The adsorption (k_{on}) and desorption (k_{off}) rate constants collectively determine the equilibrium constant for the interface. The areal density of binding sites at the interface between the phosphocholine and solid support surface are assumed to be constant throughout the FRAP experiment. Because the absolute surface density of sites is not known and may vary slightly depending on the details of surface preparation, the data can be treated in the context of a single model by expressing the concentrations of F , M and B in terms of fractional monolayer coverage. The normalization of surface concentrations to monolayer coverage allows the definition of a pseudo-on rate constant $k_{on}^* = k_{on} \cdot M$. The steady state concentration of each species (F , M , and B) can be determined from the functional form of the FRAP recovery. The time constant for diffusion τ_D , k_{on}^* and k_{off} were determined using a protocol for data processing that has been described elsewhere.[20, 21] We recap the salient features of the model briefly below.

Bilayers with calcium and without were normalized using the Phair double normalization method[17] and fit using the full reaction diffusion model:

$$frap(t) = \mathcal{L}^{-1} \left\{ \frac{1}{p} - \frac{F_{eq}}{p} \cdot [1 - 2K_1(q\omega) I_1(q\omega)] \cdot \left(1 + \frac{k_{on}^*}{p + k_{off}} \right) - \left(\frac{B_{eq}}{p + k_{off}} \right) \right\} \quad (2)$$

$$q = \sqrt{\left(\frac{\omega^2 p}{\tau_b} \right) \left(1 + \frac{k_{on}^*}{p + k_{off}} \right)} \quad (3)$$

where $frap(t)$ is recovered by taking the inverse Laplace transform ($\mathcal{L}^{-1}(x)$) of Eq. 2. F_{eq} and B_{eq} represent the free fraction, also known as the ‘mobile fraction’, and bound fraction, respectively. The mobile fraction and bound fractions are related to k_{on}^* and k_{off} through Eq. 4. ‘ K_1 ’ and ‘ I_1 ’ are the modified Bessel functions, ω is the radius of the bleaching spot, ‘ q ’ is described by Eq. 3, a ‘ p ’ is the complex variable related to time through the Laplace transform.

$$F_{eq} + B_{eq} = 1$$

$$F_{eq} = \frac{k_{off}}{k_{on}^* + k_{off}}, B_{eq} = \frac{k_{on}^*}{k_{on}^* + k_{off}} \quad (4)$$

For each surface, recoveries were collected over a range of bleaching spot sizes (radius = 5 – 20 μm , 5 μm increments).

For a homogeneous 2D film, translational diffusion is described in the context of Brownian motion (Eq. 6).[22]

$$D_T = \frac{\langle \omega^2 \rangle}{4\tau_b} \quad (6)$$

For each bleach spot size measurement, the time constants τ_b derived from the full reaction model were plotted against the square of the given bleach spot size ' ω .' The diffusion constant D_T , binding equilibrium constants K_{eq} , and calculated free energy associated with binding and diffusion, G^0 , for films containing the tethered probe NBD-LPC at the phosphorylated interface (NBD-LPC/ PO_3), calcium modified interface (NBD-LPC/ Ca/PO_3), and films containing the noninteracting perylene probe at the phosphorylated interface (P/PO_3) as a function of overlayer pH are shown in Table 2. Of particular interest is the dependence of these interfacial properties on the pH of the aqueous overlayer.

These results provide, collectively, a body of information that gives substantial insight into the chemical interactions responsible for the interfacial properties. The pH-dependencies of the interface dynamics vary with the specific system, but the dependence for the NBD-containing bilayers requires that interactions at the bilayer-support interface are the functionalities affected by pH (Fig. 5). Bilayers containing perylene as the diffusional probe provide control information on the bilayer properties where the probe is not bound to individual adlayer constituents. For this system, there is no observed pH dependence. This result provides two pieces of information. The first is that the diffusion dynamics of an untethered probe are largely decoupled from the dynamics of the bilayer constituents (*vide infra*). The second significant conclusion is that, because there is no observed pH-dependence of the perylene diffusion dynamics, changes in overlayer pH have little effect on the organization of the bilayer structure. This finding is significant because the outer leaflet headgroups are phosphocholine functionalities and their charge state is known to be pH-dependent.

The diffusional dynamics of the tethered chromophore do exhibit a pH-dependence (Table 2) and it is this dependence that relies on the interactions between the bilayer inner leaflet and the support surface (Fig. 5). These results demonstrate a more strongly pH-dependent D_T for tethered NBD for the Ca^{2+} -containing interface than for the interface that does not contain Ca^{2+} . For pH 3 and 7.5, there is a measurable difference between the interfaces but the result is the same for the two interfaces at pH 5. We understand this effect in terms of the species present at the interface.

While the k_a values for the surface phosphate functionalities are not well characterized, we take their values to be similar to those of methylene diphosphonate (MDP: $\text{p}K_{a1} = 1.70$, $\text{p}K_{a2} = 2.75$, $\text{p}K_{a3} = 7.33$, $\text{p}K_{a4} = 10.42$) and the DMPC value to be similar to that observed in the bulk ($\text{p}K_{a1} \sim 1$).[23, 24] For pH 3, both the fully protonated and mono deprotonated species

are present, in approximately equal amounts. The species present is almost exclusively the singly deprotonated form at pH 5. At pH 7.5, both the fully deprotonated and mono deprotonated species are present, in approximately equal amounts. We present the alpha fraction plot for DMPC and MDP in Fig. 6. From the known MDP pK_a values, and the formation constants for the MDP- Ca^{2+} complexes bound via a single phosphonate ($K_f = 6.5$) and bridged between two phosphonates ($K_f \sim 3.0$) a plot of the relative charge vs. pH is shown (Fig. 7).[23] This plot shows the difference between the charge experienced by the DMPC headgroups at the measured pH values. The interface-*independent* D_T at pH 5 is likely due to charge neutralization. The interface-*dependent* D_T values at pH 3 (0.9e) and 7.5 (0e) require further consideration and are discussed below.

The interface-dependent D_T data at pH 3 are attributed to the stoichiometry of the $Ca-PO_3$ interaction. For the Ca^{2+} containing surface, the presence of H^+ allows for the competitive binding between $Ca-PO_3$ (stoichiometry not specified) and the $Ca-DMPC$ equilibria. Previous studies have shown that at pH values <4 , the protonation state of the phosphoric acid moiety on the DMPC headgroup contributes significantly to overall membrane charge. [25, 26] The resulting addition of H^+ to an increasingly positively charged DMPC interface in the presence of desorbed Ca^{2+} allows for competitive complexation between the DMPC- Ca^{2+} and surface PO_3-Ca^{2+} . The large increase seen for the calcium containing interface is attributed largely to the increased increase in hydration of the interlayer between the supporting surface and DMPC interface. The desorption and subsequent hydration of the surface phosphonates requires additional hydration waters for the calcium. Addition of H^+ to interface without calcium leads to the fully protonated $PO(OH)_2$ surface where D_T is mediated predominantly by hydrogen bonding interactions.[16]

The potential explanation for the data at pH 7.5 is based on the principle of macroscopic charge neutrality. At $pH > 5$, the neutralized interface begins to accumulate negative charge due to the increased OH^- concentration. The overall increase in D_T for both interfaces with and without calcium is likely due to a decrease in surface interaction between the DMPC and support or possibly partial desorption of lipid leaflet away from the interface.[27, 28] The increased pH leads to complete neutralization of the supporting interface due to $Ca^{2+}-PO_3^{2-}$ complexation. The increased electrostatic repulsion due to increased OH^- may be the driving force for an increased D_T . The lower D_T exhibited in the absence of Ca^{2+} may be due to a combination of the interaction between the positively charged choline moiety on the DMPC headgroup and the negatively charged phosphorylated surface or a subsequent increase in viscosity of the confined water layer through increased hydrogen bonding with the supporting surface.[29]

Van't Hoff analysis reveals enthalpically driven dynamics

The next issue of importance is the strength of interaction between the bilayer phosphocholine headgroups and the phosphorylated surface. The values of K_{eq} are extracted from the FRAP data and when used to extract thermodynamic information with the aid of van't Hoff plots (Fig. 8), it is seen that the relationship between $\ln(K_{eq})$ and T^{-1} (between 25–50°C) is not linear, as expected. This is not a surprising result, because the phosphocholine DMPC exhibits a bulk gel-to-fluid phase transition of the lipid acyl chains at

24 °C.[24, 30] While the van't Hoff presentation of the data are expected to be linear for a single-phase system, a break in the relationship is a natural consequence of the phase transition. In systems such as this, the functional forms of the van't Hoff curves can be fit to extract thermodynamic information (Table 3).[31, 32]

The values reported for K_{eq} and G^0 reveal no pH-dependence for the phosphorylated support interacting with the phosphocholine headgroups in the absence of Ca^{2+} . The interactions for all pH values are likely those between the cationic choline functionality from the phospholipid and the (anionic) phosphate functionality of the support surface. The situation is markedly different with the addition of Ca^{2+} . At pH 3 the interaction becomes favorable, and we attribute this to the favorable competition of either the surface-bound phosphate or the phosphocholine headgroup phosphate for Ca^{2+} in an environment characterized by very low $[OH^-]$. For higher pH, where there is more OH^- present, even if at a low level, the competition for Ca^{2+} is poised more in favor of OH^- complexation.

It is useful to note that for the case of perylene diffusion, the FRAP measurements remain sensitive to the dynamics of both the probe and the bilayer constituents. For perylene diffusion, there is a discernible pH-dependence to the bilayer-surface binding, consistent with that seen for the tethered probe, where the connection between surface binding and translational diffusion is more intuitive.

Conclusions

The dynamics of a simple lipid bilayer supported on a planar solid (silica) that has been modified to contain phosphate functionality is found to depend on the pH of the aqueous overlayer, and on the presence or absence of Ca^{2+} . The dynamics of the bilayer system are compared for both free and tethered chromophores and the results are consonant. An important conclusion of the pH-dependent data we report here are that the bilayer is porous enough on the molecular scale to reveal pH-dependent interactions between the bilayer and the modified support. In all cases the interactions between the bilayer and support are modest in strength, rendering the interfaces as fluid entities. This is not a surprising result, but one that remains to be explored fully in the context of how mono- and bilayer fluidity can be controlled by means of the chemical functionality present on the support.

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Figure 1.

Idealized diagram of the formed bilayer structure at the phosphorylated silica support interface. The prepared samples are all comprised of a phosphorylated silica support, DMPC bilayer, and aqueous adlayer of varying pH. The DMPC bilayers containing the fluorescent probe NBD-LPC with calcium and without are shown in (a.) and (b.), respectively. The DMPC bilayers containing perylene and without calcium are shown in (c.). Hot spots containing varying degrees of negative charge are shown as red circles, varying degree of positive charge shown as yellow circles.

Figure 2.

XPS survey scans of the phosphorylated support surfaces without (PO_3^{2-}) and containing Ca^{2+} ($\text{PO}_3^{2-}/\text{Ca}^{2+}$). Inset shows baseline shifted detailed scan of the O1s peak shift.

Figure 3.

Normalized FRAP curves for membranes containing the tethered NBD-LPC chromophore supported on surfaces containing calcium (a) and associated FRAP micrographs vs. pH for a 10 μm bleaching radius at times before bleaching ($t < b$), immediately after bleaching ($t = b$), and 10s and 60s after bleaching (b). Best-fit curves to the reaction-diffusion model are shown.

Figure 4.

Normalized FRAP curves for membranes containing the tethered NBD-LPC chromophore supported on surfaces without calcium (a) and associated FRAP micrographs vs. pH 10 μm bleaching radius at times before bleaching ($t < b$), immediately after bleaching ($t = b$), and 10s and 60s after bleaching (b). Best-fit curves to the reaction-diffusion model are shown.

Figure 5. Translational diffusion coefficient (D_T) of the tethered (NBD-LPC) and non-tethered (perylene) probes for the phosphorylated surfaces with and without Ca^{2+} under varying adlayer pH conditions. Error bars indicate calculated uncertainty from the fits using Eq. 2.

Figure 6.

Calculated alpha fractions for methylene diphosphonate (red lines) and for DMPC (blue lines) as a function of pH. Data are calculated with the assumption that the dissociation constants for surface phosphate functionality is similar to those for methylene diphosphonate.

Figure 7.

Calculated relative charges for DMPC (black), surface phosphates (red), and calcium-surface phosphate complex (blue) are shown. The relative charge on the surface phosphates (red), and consequently the total surface charge on the films without calcium is calculated with the assumption that the dissociation constants for surface phosphate is similar to those for methylene diphosphonate. The total interfacial charge for the calcium containing films is shown (green dash).

Figure 8.

Van 't Hoff plot of $\ln(K_{eq})$ vs. inverse temperature for the tethered (NBD-LPC) film without Ca^{2+} at pH 3, 5 and 7.5. Lines are best-fit polynomials (2nd degree) to the Van 't Hoff equation.

Table 1

Ellipsometric thickness of the spin-coated DMPC bilayers

	P/PO₃ (Å)	NBD/PO₃ (Å)
Ambient	51.0 ± 2.2	47.4 ± 2.0
Aqueous Adlayer	51.5 ± 2.8	47.5 ± 2.2

Errors in parentheses represent standard deviation ($\pm 1\sigma$) between two independently prepared samples and 3 measurements on each sample

Table 2

Determined translational diffusion coefficient ' D_T ', equilibrium binding constant, ' K_{eq} ', and Gibbs free energy for the reaction-diffusion process ' G^0 '.

	System	pH		
		3.0	5.0	7.5
D_T ($\mu\text{m}^2/\text{s}$)	NBD/ PO_3	2.20 $\pm$ 0.24	1.79 $\pm$ 0.13	2.74 $\pm$ 0.08
	NBD/Ca/ PO_3	7.23 $\pm$ 0.45	1.81 $\pm$ 0.17	3.92 $\pm$ 0.25
	P/ PO_3	2.25 $\pm$ 0.05	2.20 $\pm$ 0.15	2.29 $\pm$ 0.24
K_{eq}	NBD/ PO_3	0.39 $\pm$ 0.12	0.27 $\pm$ 0.11	0.39 $\pm$ 0.11
	NBD/Ca/ PO_3	1.42 $\pm$ 0.32	0.28 $\pm$ 0.10	0.60 $\pm$ 0.13
	P/ PO_3	0.10 $\pm$ 0.02	0.19 $\pm$ 0.07	0.28 $\pm$ 0.07
G^0 (kJ/mol)	NBD/ PO_3	2.41 $\pm$ 0.87	3.36 $\pm$ 1.38	2.39 $\pm$ 0.69
	NBD/Ca/ PO_3	-0.66 $\pm$ 0.29	3.17 $\pm$ 1.07	1.27 $\pm$ 0.27
	P/ PO_3	6.27 $\pm$ 1.41	4.70 $\pm$ 1.66	3.48 $\pm$ 0.92

Uncertainties are reported as standard deviations ($\pm 1\sigma$) between two independently prepared samples and a minimum of 20 measurements on each sample (5 measurements at each bleaching spot size).

Table 3Calculated thermodynamic parameters for the NBD/PO₃ films from Van't Hoff analysis at 298.15 K

pH	3.0	5.0	7.0
H (kJ/mol)	102±1	133±2	81.2±1.2
S (kJ/mol-K)	0.340±0.002	0.436±0.005	0.265±0.004
G ⁰ (kJ/mol)	2.33±0.70	3.21±2.34	2.35±1.60

Uncertainties are reported as standard deviations ($\pm 1\sigma$) derived from polynomial regression of a minimum of three measurements at each temperature.